PHOTOREVERSIBLE CHANGES IN HYDROPHOBICITY OF UNDEGRADED PEA PHYTOCHROME DETERMINED BY PARTITION IN AN AQUEOUS TWO-PHASE SYSTEM

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1. Introduction

Phytochrome is a chromoprotein with two distinct and photointerconvertible forms, a red-light-absorbing form, P_r, and a far-red-light-absorbing form, P_{fr} [1]. Phytochrome acts as the transducer for a variety of photomorphogenetic responses in plants, where P_{fr} is active and P_r is inactive [2]. Thus, information concerning differences in molecular properties between P_r and P_{fr} may help to elucidate the mechanism of biological action of phytochrome.

P_{fr} has been shown to be qualitatively more hydrophobic than P_r using 'hydrophobic' chromatography [3] and a fluorescent probe [4]. Phototransformation resulted in a very limited change in molecular structure of phytochrome as indicated by studies on intrinsic fluorescence and circular dichroism [5] and by chemical modification of phytochrome [6]. The photoinduced change in hydrophobicity of the phytochrome molecules might be functionally important in its biological action.

In general, hydrophobicity of smaller molecules is determined in terms of their solubility in organic liquids [7]. This method is not applicable to measurement of hydrophobicity of proteins in their native conformation because of the denaturing effect of organic solvents on them. Instead hydrophobic interactions of proteins with fatty acyl chains have been used as a measure of hydrophobicity [8]. The hydrophobic interactions can be determined by partition in an aqueous two-phase system containing dextran and poly(ethylene glycol) (PEG) and poly(ethylene glycol)-palmitate (PEG-P) without the complication due to the presence of organic solvents, because the system is rich in water.

Here, we have applied this method for purified pea phytochrome to determine quantitatively the difference in hydrophobicity between: (i) P_r and P_{fr}; and (ii) undegraded and degraded phytochrome. We discuss the possible role of the photoreversible change in hydrophobicity in the binding of phytochrome to particulate fractions.

2. Materials and methods

About 30% pure phytochrome was obtained from 7-day-old etiolated seedlings of *Pisum sativum* cv. Alaska by ammonium sulfate fractionation, brushite and DEAE-agarose (DEAE Bio-gel, Bio-Rad Labs) chromatography [9]. The method for further purification of this sample was newly developed as follows: ~20 mg partially purified phytochrome was dissolved in 350 mM KCl, 14 mM 2-mercaptoethanol and 10 mM K-phosphate (pH 7.8). After exposure to 2.6 W/m^2 red light for 5 min, the phytochrome solution was applied to a 1.5 × 3.5 cm column of ω-aminooctyl agarose (Miles Labs) pre-equilibrated with the same buffer under red light. The column was washed with 6 column vol. of the starting buffer under a dim-green safe light. The column was then irradiated with 2.4 W/m^2 far-red light for 15 min, and eluted with the same buffer. Fractions eluted after the far-red light irradiation contained 95% pure undegraded phytochrome judged from Coomassie brilliant blue-staining of SDS gel electrophoresis [10]. Phytochrome eluted before the far-red light irradiation was collected by ammonium sulfate precipitation and purified with ω-aminooctyl agarose chromatography again. The yield of phytochrome with each ω-aminooctyl agarose chromatography was ~40%.

The tryptic peptide of phytochrome was also

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obtained; the M_r of this peptide was 60 000 (60 kM_r fragment) and its photoreversible absorbance changes were similar to those of undegraded phytochrome. The peptide was prepared by incubation of 90% pure undegraded phytochrome in 100 mM Na-phosphate (pH 7.8), 1 mM Na_2EDTA and 14 mM 2-mercaptoethanol with L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington) at 1:400 (w/w) at 20°C for 30 min, followed by the addition at 0.2 mM final conc. of phenylmethanesulfonyl fluoride, and filtration on a 1.6 × 81 cm column of Sephacryl S-200 (Pharmacia) equilibrated in 100 mM Na-phosphate (pH 7.8), 1 mM Na_2EDTA and 0.5 mM dithiothreitol. Purity of the 60 kM_r fragment was 85% as measured by Coomassie brilliant blue-staining of SDS gel electrophoresis [10]. Major contaminants in the preparation of the 60 kM_r fragment were peptides of 55 000 and 46 000 M_r, respectively. They accounted for 10% of all the staining. We did not detect any peptides of $M_r > 60\,000$ in the preparation. All the procedures were carried out entirely in darkness or under a dim-green safe light at 0–4°C except when described otherwise.

Bovine serum albumin (BSA) and cytochrome *c* from Sigma and dextran T-70 ($\bar{M}_r$ = 70 000) from Pharmacia were used without further purification. PEG 6000 ($\bar{M}_r$ = 7500) from Wako Pure Chemicals was used after purification twice by recrystallization from acetone. PEG-P was synthesized from the purified PEG and palmitoyl chloride [11].

The degree of hydrophobic interactions between proteins and $C_{16:0}$ fatty acyl chains were determined by measuring their partition in an aqueous two-phase system containing dextran and PEG and PEG-P according to [8]. The system used here was composed of 8% (w/w) dextran, 8% (w/w) PEG including its palmitic acid ester, and 10 mM K-phosphate (pH 7.2). The aqueous two-phase system separates into a PEG and PEG-P-rich upper phase and a dextran-rich lower phase. The free energy ($\Delta G'$) required to transport 1 mol solute from the lower to the upper phase in the system was calculated as $\Delta G' = -RT \ln C_u/C_l$, where R, T, C_u and C_l are gas constant, absolute temperature, concentrations of the solute in the upper and the lower phase at equilibrium, respectively. The concentration of the solutes was determined by their amino group assay using fluorescamine [12]. The free energy (ΔG) required to transport 1 mol solute from the upper phase in a system without PEG-P to the upper phase in a system containing PEG-P was obtained as $\Delta G = \Delta G'' - \Delta G'$, where $\Delta G'$ and $\Delta G''$ are the free energy for the transportation from the lower to the upper phase in the system without PEG-P and in the PEG-P containing one, respectively. Since the electrostatic term in the $\Delta G'$ and $\Delta G''$ is cancelled by the subtraction [8], ΔG can be a measure of the degree of hydrophobic interactions between the solute and the fatty acyl chains. Here, $-\Delta G$ is used and a larger value indicates stronger interactions. The results for BSA and cytochrome *c* shown in fig.1 were essentially the same as in [8], indicating that this two-phase system was comparable to that in [8].

Red light was provided by 6 20 W fluorescent tubes (Toshiba FL20SW) behind 3 mm thickness of a red acrylic resin sheet (Shinkolite A, Mitsubishi Rayon Co.). Far-red light was provided by 6 20 W fluorescent tubes (Toshiba FL 20SFR-74) behind a black plastic film (IRP-1, NEC) and the red acrylic resin sheet. The dim-green safe light was obtained from 40 W fluorescent tubes (Toshiba FL20SW) wrapped with dark-green plastic film (Filmolux, color no. 087, Hans Neschen GmbH).

3. Results

The partition coefficient of undegraded phytochrome in an aqueous two-phase system containing various concentrations of PEG-P was determined under red or far-red light. $-\Delta G$ of the phytochrome obtained from values of the partition coefficient increased under both light conditions as the concentration of PEG-P was increased (fig.1).

We measured the absorption spectra of phytochrome in the upper and lower phases of systems containing various concentrations of PEG-P. The degree of photoreversibility of the absorbance change decreased as the PEG-P concentration increased, and the loss of photoreversibility was greater in the PEG and PEG-P-rich upper phase (fig.2). In the case of 1% substitution by PEG-P, however, the photoreversibility remained >93% in both the upper and the lower phases. Therefore, $-\Delta G$ was obtained under these conditions.

The results (table 1) show that undegraded phytochrome under far-red light irradiation has 38% as much $-\Delta G$ as that of BSA. All of the phytochrome was in the P_r form under this light condition. The value increased markedly on red light irradiation. Compared with undegraded phytochrome, the

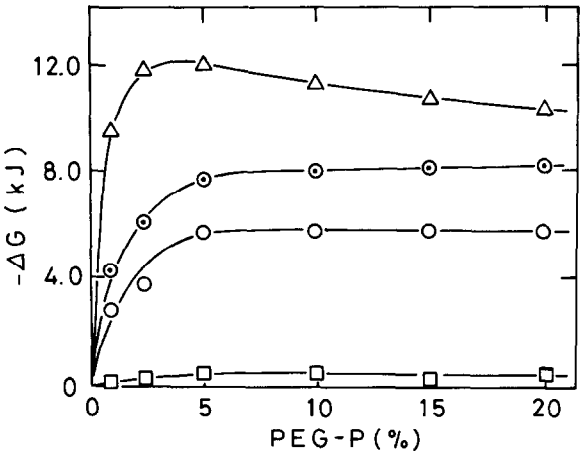

Fig.1. The effect of varying the concentration of PEG-P in an aqueous two-phase system, on the partition of undegraded phytochrome under far-red light (○) and under red light (⊙), bovine serum albumin (△) and cytochrome *c* (□). An aqueous two-phase system contained 8% (w/w) dextran, 8% (w/w) PEG plus PEG-P, 10 mM K-phosphate (pH 7.2), and a substrate. After addition of 0.2 g 10 mM K-phosphate (pH 7.2) containing undegraded phytochrome to 1.8 g of the phase system solution, the total solution was irradiated with red (1.8 W/m²) or far-red (2.4 W/m²) light for 10 min and then mixed gently with a vortex mixer under the same light. It was centrifuged at 600 × *g* for 20 min to enhance the separation under the dim-green safe light. The final protein concentrations were 34 μg/mg for undegraded phytochrome and 50 μg/mg for the other proteins. Partition between the two phases is expressed as $-\Delta G$ at 22°C.

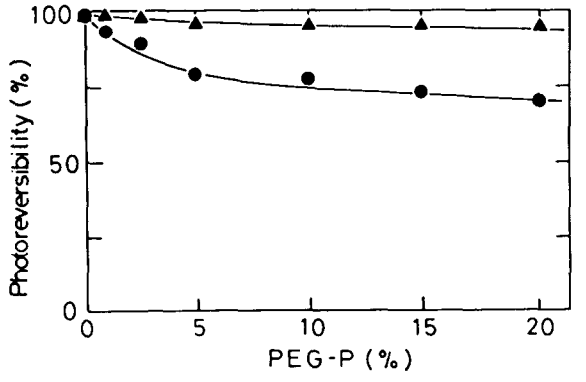

Fig.2. The effect of varying the concentration of PEG-P in an aqueous two-phase system on the photoreversibility of undegraded phytochrome in the upper (●) and lower phase (▲) at 22°C. Photoreversibility was defined as % recovery of A_{666} of phytochrome after 660 nm and subsequent 735 nm irradiation. The photoreversibility was determined by measuring absorption spectra of the phytochrome in each phase of the solution at 34 μg/mg using a Hitachi 557 spectrophotometer.

Table 1
Partition in an aqueous two-phase system expressed as $-\Delta G$ at 22°C

Substrate	$-\Delta G$[a] (kJ)
Undegraded phytochrome	
under far-red-light irradiation	3.39 ± 0.38
under red-light irradiation	4.85 ± 0.46
60 kM_r fragment of phytochrome	
under far-red-light irradiation	0.96 ± 0.17
under red-light irradiation	1.13 ± 0.13
Bovine serum albumin	8.91 ± 0.54
Cytochrome *c*	0.13 ± 0.08
Glycyl–glycine	0.13 ± 0.04
Glycyl–leucine	−0.04 ± 0.04
Glycyl–phenylalanine	0.04 ± 0.13

[a] Partition coefficients were obtained as in fig.1. The fraction of PEG-P in the PEG-P-containing system was 1% (w/w). The final concentrations of undegraded phytochrome and 60 kM_r fragment were 34 μg/mg and 18 μg/mg, respectively, or 0.3 nmol/mg for both proteins assuming the M_r-values of undegraded phytochrome and the 60 kM_r fragment to be 114 000 and 60 000, respectively [9]. The values of $-\Delta G$ are the average of 3 measurements, except for the phytochrome determinations which are the average of 5 measurements

60 kM_r fragment exhibited a smaller $-\Delta G$ value for P_r and no increase of $-\Delta G$ on red light irradiation. The photoreversibility of the 60 kM_r fragment was 98% in this condition. $-\Delta G$ was almost zero within the errors for amino acids, glycine, leucine and phenylalanine (not shown), and dipeptides.

4. Discussion

This study demonstrated that hydrophobicity of undegraded phytochrome, as determined in terms of its affinity for $C_{16:0}$ fatty acyl chains, changed remarkably during the photoreversible transformation of phytochrome between P_r and P_{fr} (fig.1,2). Undegraded phytochrome in P_r form showed 38% as much hydrophobicity (table 1) as that of BSA which is known as a fatty acid carrier [13]. The hydrophobicity increased up to 54% of the BSA-value under red light irradiation (table 1) indicating that P_{fr} is more hydrophobic than P_r. Interactions between proteins and hydrophobic ligands such as the palmitoyl-group, requires the presence of a hydrophobic domain of appropriate size in the proteins [7]. In fact, small molecules, such as amino acids or dipep-

tides can not interact with the hydrophobic ligand (table 1). Relatively large $-\Delta G$ of P_{fr} of undegraded phytochrome suggests the presence of such hydrophobic domain in it.

The 60 kM_r fragment of phytochrome, on the other hand, was less hydrophobic than the undegraded phytochrome in the P_r form and showed no increase of hydrophobicity upon phototransformation (table 1). These facts suggest that phytochrome is a multifunctional protein [14] which has a chromophoric domain and a hydrophobic domain; and it is probable that the hydrophobic domain responsible for the photoinduced hydrophobicity increase is separated by trypsin digestion from the chromophoric domain which contains a chromophore(s) and exhibits phototransformation between P_r and P_{fr}. It has been shown that undegraded rye phytochrome binds agarose-immobilized Cibacron blue 3GA [15], but that the 60 kM_r fragment of the phytochrome does not [16]. Since the interaction between the undegraded phytochrome and the blue dye was rather hydrophobic [15], the binding domains for the blue dye and for the palmitoyl group shown in the present study could be the same.

We have shown that P_{fr} of undegraded phytochrome binds to microsomes in vitro in the presence of Ca^{2+}, but that P_{fr} of the 60 kM_r fragment does not [9]. This fact suggests that the red-light-induced increase of hydrophobicity of the undegraded phytochrome might cause the binding of phytochrome to biomembranes [17]. Since the binding of phytochrome could be a primary biological action of phytochrome [18], further characterization of the photoinduced hydrophobicity change of phytochrome is required.

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